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DETERMINING UPPER LIMIT OF PHYTOTOXICITY OF SUGAR BEET CV. DIAMENTA, POLYGERM LINE EMU 8, AND MONOGERM LINE SG3 AGAINST KANAMYCIN SULFATE AND PHOSPHINOTHRICIN

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This study reports upper limit of phytotoxicity (LD_{100}) levels of three sugar beet genotypes – monogerm cv. Diamenta, polygerm line EMU 8, and monogerm line SG3 to pre screen them for use in genetic transformation studies against Kanamycin sulfate and phosphinothricin on MS medium containing either of these supplemented with 30 g/l sucrose. The results suggest that 300 mg/l kanamycin and 2.5 mg/l phosphinothricin was the most optimum dose to kill these genotypes. These concentrations were determined before carrying out genetic transformation studies to avoid escapes. The results of the study are assumed to facilitate in genetic transformation through direct seed culture.
Keywords: sugar beet, genetic transformation, optimization, lethal dose.

INTRODUCTION

Sugar beet (*Beta vulgaris*) genus Spinacia, a biennial, diploid (2n = 18) plant is used as main source of sugar in Turkey and has considerable production in 59 out of 81 provinces (Kahriz, 2016). The first gene transfer was carried out in 1983 with the *Agrobacterium tumefaciens* (Caplan *et al.* 1983, Zambryski *et al.* 1983). Although transformation of sugar beet is highly problematic due to recalcitrant behavior of plants towards regeneration, yet number of successful transformation studies have been reported in the literature (Türkşeker, 2015, Pourali Kahriz, 2016). Production of transgenic plants involve use of a selectable marker genes (*bar* and *npt II*) to favor regeneration of transformed shoots and plants. The regenerated plants or shoots are generally selected on Kanamycin sulfate or phospinothricin; or other less commonly used selection agents. Both *bar* and *npt II* genes provide resistance against phosphinothricin and kanamycin (Bowen, 1993). Information about upper limit of phytotoxicity (LD_{100}) levels of the selection agents before actual genetic transformation studies help in making the transformation easier and avoid escapes. Although number of transformation procedures have been reported for sugar beet world over, transformation studies are rare in Turkey. Information regarding the magnitude and upper limit of toxicity of these selection agents are also missing.

The aim of this study was to identify the upper level of phytotoxicity (LD_{100}) of three sugarbeet genotypes commonly used in Turkey against Kanamycin sulfate and phosphinothricin.

MATERIALS AND METHODS

The seeds of monogerm cv. Diamenta type, polygerm line EMU 8, and monogerm line SG3 were obtained from the Sugar Beet Research Institute Ankara Turkey.

The Sugar beet seeds are irregular and serrated in shape with hard-shelled, pericarp and the embryo lies in the fruit / seed cavity. These were sterilized as described by (Kahriz and Kahriz, 2017)

Lethal dose$_{100}$ (LD_{100}) for each of the above mentioned genotypes was determined by germinating their seeds using selection medium containing 50, 100, 150, 200, 250, 300 and 350 mg/l kanamaycin and 0.5, 1, 1.5 and 2 mg/l phosphinothricin in agar solidified MS medium containing 30 g/l sucrose to facilitate the future transformation work using *Agrobacterium tumefaciens* strain LBA 4404 ::pRGG bar that provides resistance against both kanamycin and phosphinothricin.

The germinated seedlings remained on the selection medium for 15 days. Thereafter, the number of living plants were counted and the percentage was calculated using formula mentioned below

$$\text{Percentage} = \frac{\text{number of living plants}}{\text{total number of plants}} \times 100$$

Each treatment contained 400 seeds equally divided into 8 replications containing 50 seeds/replication.

RESULTS

***Determination of upper level of phytotoxicity using kanamycin sulfate*:** The results of the study showed 100% living seedling on 50 mg/l kanamycin throughout irrespective

Table 1: Determining upper level of phytotoxicity (LD_{100}) for 3 sugar beet genotypes using different concentrations of kanamycin sulphate in agar solidified MS medium for 15 days

Genotypes	Percentage (%) of living plants						
	50 mg/l km	100 mg/l km	150 mg/l km	200 mg/l km	250 mg/l km	300 mg/l km	350 mg/l km*
Diamenta	100	95.63	63.41	48.23	35.26	10.37	0.00
Emu 8	100	90.73	60.51	42.44	30.28	9.11	0.00
SG3	100	82.68	51.46	37.34	29.34	5.67	0.00

* kanamycin sulphate

Table 2: Determining upper level of phytotoxicity (LD_{100}) for 3 sugar beet genotypes using different concentrations of phosphinothricin in agar solidified MS medium for 15 days

Genotypes	Percentage (%) of living plants					
	0.5mg/l ppt	1mg/l ppt	1.5 mg/l ppt	2 mg/l ppt	2.5mg/l ppt	3 mg/l ppt*
Diamenta	99.00	96. 73	72.70	15.11	0.00	0.00
Emu 8	95.12	91.82	80.32	33.25	0.00	0.00
SG3	98.51	93.91	79.11	21.72	0.00	0.00

*phosphinothricin

of the genotype used in the study. However, each increase in the concentration of kanamycin in the selection medium showed increased rate of mortality on the germinating seedlings. Living seedlings (escapes with natural resistance against kanamycin) percentage on 100, 150, 200 and 250 and 300 mg/l kanamycin ranged 82.68 – 95.63%, 51.46 – 63.41%, 37.34 – 48.23%, 29.34 – 35.26%. and 5.67 - 10.37% respectively.

The most affected genotype was SG3 and the least affected genotype was cv. Diamenta in each case.

Complete mortality was observed in a short time (within 5-7 days) on all genotypes selected on 350 mg/l kanamycin in the culture medium.

This dose of kanamycin was used to select all sugar beet plants for selection against *npt II* gene using *A. tumefaciens* strain LBA 4404::pRggbar.

***Determination of upper level of phytotoxicity using phosphinothricin*:** The results showed living seedlings in range of 95.12- 99.00%, 91.82 -96.73%, 72.70 – 80.32%, 15.11 – 33.25% on 0.50, 1, 1.5, 2 and 2.5 mg/l phosphinothricin respectively.

The most affected genotype was EMU8 and the least affected genotype was Diamenta in each case.

Total Mortality was noted within 4-8 days on all genotypes on 3.00 and 3.50 mg/l phosphinothricin in the culture medium. Therefore, 3.00 mg/l phosphinothricin was used to select all sugar beet genotypes during genetic transformation studies using *A. tumefaciens* strain LBA 4404::pRgg bar..

DISCUSSION

Bioassays reveal variations related to the phenotypic responses of a single or several live tissues in medium containing slection agents. The main focus of LD_{100} toxicity test should be total motility of the plants during pre-transformation studies.

Kanamycin resistance in many plant species is achieved by transformation of the plants with *npt II* gene. Non natural resistant seedlings showed variable necrosis depending on the level of kanamycin in the selection medium that inhibited activity of glutamine synthase (Bayer *et al.* 1972; Tachibana *et al.* 1986) and synthesis of of plastid protein [Gray *et al.* 1984) with impairment of lipid reserve mobilization in 2-3 days in the presence of sucrose (Penfield *et al.* 2004) variably in dose dependent manner. It is assumed that this activity was maximum at 300 mg/l kanamycin in the selection medium; where total mortality was noted on all germinating seedlings.

Glufosinate or phosphinothricin resistance in many plant species is achieved by transformation of the plants with *bar* gene derived from *Streptomyces hygroscopicus*, placed under the control of a CaMV 35S (Hérouet *et al.* 2005).

One of the secondary effects of the phytotoxic response to glufosinate is reduced photosynthetic ETRs as has been noted in white mustard (Ziegler and Wild, 1989), soybean (Barberis, 2012) and cucumber plants (Dayan and Zaccaro, 2012) treated with glufosinate. The extremely rapid acetylation of L-phosphinothricin into non-toxic *N*-acetyl-L-phosphinothricin metabolite achieved in glufosinate-resistant IMACD 6001LL (Droge et al. 1992) protected this cultivar from inhibition of rubisco enzyme activity and overall photosynthetic activity.

Application of glufosinate (phosphinothricin) causes ammonia accumulation in most plant species, including *Sinapis alba* and maize (Wendler *et al.* 1990), *Setaria viridis* and barley (Mersey *et al.* 1990), *Tripleurospermum inodorum* (Manderscheid *et al.* 2005) and rice (Tsai *et al.* 2006).

It is assumed that every plant species including sugar beet has resistance against phosphinothricin that do not allow increment in ammonia levels in plants at lower doses of the

chemical and this resistance increase rate of escapes during transformation if they are not carefully selected pre transformation. The lack of ammonia accumulation at low levels of phosphinothricin selection indicates that GS activity is not damaged (Manderscheid and Wild 1986) and must be determined empirically for each plant species to be transformed.

It was noted that 2.5 mg/l phosphinothricin was sufficient to cause sufficient injury to the sugar beet genotypes used in the study and could be safely used to select the transgenic plants in agreement with (Sweeney and Jones 2015).

***Conclusıon*:** The information reported here may facilitate *Agrobacterium-mediated* transformation of sugar beet cultivars through seeds.

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